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Thermostabilization by the Improvement of Intertrimeric Residues in *Thermus thermophilus* Inorganic Pyrophosphatase

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ABSTRACT

Inorganic pyrophosphatase (EC. 3.6.1.1) from *Thermus thermophilus* (*Tth* PPase) is a thermostable homohexamer of 174 amino acids, and its intertrimer interface is formed mainly by the symmetric α-helix A between subunits. Amino acids and their interactions composing intertrimer interface are different in hexameric Family I PPases, and then it was deduced that *Tth* PPase showed high thermostability because of stabilizing this interface by interactions of these residues. In this study, we focused on Thr138 and Ala141 residues in intertrimer interface of *Tth* PPase to confirm the relationship between intertrimeric residues and thermostability, and then improved their combination to His and Asp/Glu (HD or HE variant).

As results, the HD variant showed the highest thermostability of enzyme activity, fluorescence spectra, and quaternary structure in the wild type *Tth* PPase and all variants. Especially, about 38% of hexamer and almost 40% of enzyme activity were observed in HD variant after heating even at 85°C. Therefore, we suggested that the conversion to a set of ionic His138 and Asp141 at intertrimer interface had increased the thermostability of *Tth* PPase, and then suppressed its thermal aggregation.

Keywords: Inorganic pyrophosphatase; Intertrimer interface; Site-directed mutagenesis; Thermostability, Thermus thermophilus

INTRODUCTION

Oligomeric proteins are generally stabilized by multiple interactions between subunits. In contrast with monomeric and globular proteins, they may exhibit various patterns of protein denaturation, often accompanying with dissociation of oligomeric structures [1]. Meanwhile, it has been reported that some substitutions of amino acids at the subunit interface have attained to stabilize the quaternary structure by protein engineering studies successfully. Their approaches to stabilize quaternary structure are fundamentally carried out by introducing hydrophobic, hydrophilic, ionic interactions, and so on, into subunit interface [2–4].

Inorganic pyrophosphatase (PPase, EC. 3.6.1.1) is one of oligomeric enzyme which catalyzes the hydrolysis of inorganic pyrophosphate, and divalent cations such as Mg^{2+} are essential for the enzymatic activity [5]. To date, so many PPases had been reported from various source, and then comprised of two families [6]. One is Family I PPases which include three subfamilies, namely prokaryotic, plant, and animal/fungal PPases. Another is Family II PPases which are not homologous to Family I PPase, and Mn is essential for enzyme activity [7, 8]. Thus far, Family I PPases shown in Table I have been cloned, expressed and characterized in terms of thermostability, catalysis and quaternary structure [9–19]. These PPases are composed of 162–220 amino acids per subunit, and have homohexamer (dimer of trimers) or homotrimer, except for tetramer in *Thermoplasma acidophilum* PPase [19]. Moreover, the three-dimensional structures of PPases from *Thermus thermophilus* (*Tth*), *Sulfolobus acidocardarius* (*Sac*), *Pyrococcus horikoshii* (*Pho*), and *Escherichia coli* (*E.coli*) PPases had already elucidated [18,20–22]. They are similar in primary structure, the folding of three-dimensional structure, and quaternary structure, but differ in thermostability and oligomeric stability. On the basis of the investigations from three-dimensional structures, it

was deduced that the differences in thermostability might be occurred by oligomeric interactions, and trimer-trimer interface (intertrimeric interface) is likely one of candidates [18, 21, 23].

In this study, we paid attentions to the amino acids in the vicinity of intertrimeric interface of *Tth* PPase (Table I). *Tth* PPase is consists of 174 amino acids per monomer and forms thermostable homohexamer [9, 20]. It was reported that the hexamer of *Tth* PPase was comprised of two trimers, in which the trimer-trimer interface were formed from α-helix A of molecule. As shown in Table I, amino acids in the C-terminal part of α-helix A are very similar among Family I PPase except for Thr138 and Ala141 in *Tth* PPase. Most of Family I PPase adopted the combination of positive His/Arg and negative Asp/Glu residues at these positions, while that of Thr and Ala in *Tth* PPase. Concerning these observations, Leppänen *et al.* deduced that such differences in residues may change intertrimeric contact and its interactions. In *E.coli* PPase, His136'-Asp143-His140 network may stabilize the whole molecule, whereas the change from Asp143 to Glu143 presumed to occur the oligomeric shift with increased thermostability in *Sac* PPase [21]. On the other hand, since *Tth* PPase is lacking of these His140 and Asp/Glu143 interactions (They correspond to Thr138 and Ala141, respectively), it seemed to be thermostabilized in the difference ways [20, 21]. *Tth* PPase exhibits higher thermostability than *E.coli* PPase, accompanying with two remarkable features at high temperatures [9, 24]. *i.e.* (1) Hexamer of *Tth* PPase was not dissociated into trimers drastically, and (2) it was aggregated thermally followed by inactivated after heating above 85 ℃. These features are observed only in *Tth* PPase so that they prevent *Tth* PPase from thermostabilizing artificially by the protein engineering. Furthermore, we also reported that chimeric PPase between *Tth* and *E.coli* PPases had an increased thermostability owing to changes of four amino acids, Thr138, Ala141, Ala144, and Lys145 in *Tth* PPase, to those corresponding residues in *E.coli* PPase [24]. Subsequent studies in *Tth* PPase revealed that deletion of Ala144 and Lys145 suppressed thermal aggregation [25], while substitutions of Thr138 affected on the hexamer formation (Kouzai, M. *et al.*, in press). Accordingly, it is presumed that the conversion of the combination in intertrimer interface of *Tth* PPase may transform the above thermal features into more thermostable characteristics.

Therefore, we focused on intertrimeric residues, Thr138 and Ala141 in *Tth* PPase, and converted them into the combinations of His and Asp/Glu residues (intertrimer-converted variants; namely HD and HE variants). Here we report on the consequences for their characteristics and oligomeric thermostabilities for variants of *Tth* PPase, in which intertrimer residues of *Tth* PPase were converted into those of *E.coli* and *Sac* PPases, and possible designs for constructing more thermostable enzymes.

Table I. Comparison of partial amino acid sequences of wild type *Tth* PPase with those of Family I PPases.

Enzyme source	134 143
Thermus thermophilus	HFFETYKALE
Sulfolobus acidocaldarius	HFFEHYKELE
Sulfolobus tokodaii.	HFFEHYKELE
Aquifex aeolicus	HFFEHYKELE
Aquifex pyrophilus	HFFEHYKELE
Escherichia coli	HFFEHYKDLE
Bartonella bacilliformis	HFFKHYKDLE
Geobacillus stearothermophilus	HFFERYKDLQ
Thermophilic bacterium PS-3	HFFERYKDLQ
Pyrococcus horikoshii	HFFKRYKELE
Thermoplasma acidophilum	NFFSTYKILE
	*** ↑**↑*

Amino acid sequences in the vicinity of intertrimer interface of wild type *Tth* PPase are shown, compared with corresponding residues in Family I PPases. Arrows indicate examined residues in this study on wild type *Tth* PPase, and the corresponding residues in Family I PPases. Identical amino acids among PPases are indicated by asterisks. The numbers of amino acids in wild type *Tth* PPase are also shown.

MATERIALS AND METHODS

Materials and Chemicals

T4 DNA ligation Kit ver.2 and *Pyrobest* DNA polymerase were obtained from Takara Shuzo. Restriction endonucleases were purchased from Promega, New England Bio Labs and Takara shuzo. DEAE-cellulose was purchased from Wako pure chemical, and Sephacryl S-200HR was obtained from Pharmacia. *E. coli* JM109 and BL21 (DE3) strain were used for the site-directed mutagenesis and for the expression of wild type *Tth* PPase and its variants. The plasmid harboring *Tth* PPase gene was constructed as reported previously [9]. Swiss-PdbViewer ver.4.0 was downloaded from the Swiss Institute of Bioinformatics [26].

Site-Directed Mutagenesis by Polymerase Chain Reaction

Site-directed mutagenesis was performed by means of polymerase chain reaction (PCR). The genes of T138H and A141E variants were constructed as described below. The sense-primer for T138H and A141E was 5'- GTGTGGAATTGTGAGCGGATA AC -3' (23mer, corresponding to pUC118 plasmid), whereas the antisense-primers for T138H and A141E were5'-TTGGCCTCGAGGGCCTTGTAGTKTTCG AAGA-3' (31mer, corresponding to Phe135- Lys145) and 5'- CTTCCCCTTCTTGGCCTCGAGTTCCT TGTA -3' (30mer, corresponding to Tyr139 - Lys148), respectively. The amplified fragment was digested with *Xho* I and *Nco* I, and then inserted into pUCTPPCR after digestion with the same endonucleases. Then, it was sequenced, and the resultant vectors for T138H and A141E were named as pUCTPT138H and pUCTPA141E, respectively. Moreover, these vectors were digested with *Nco* I and *Bam*HI, and then each fragment was inserted into pET15b after digestion with the same endonucleases. The resultant expression vectors for variants were named as pETTPT138H and pETTPA141E, respectively. Although we also attempted to construct the expression vector for A141D variant, we failed unfortunately because of the unexpected multiple mutation so that we can not examine the characteristics of this variant. On the other hand, the genes of T138H/A141E and T138H/A141D variants were constructed by using pETTPT138H as template for PCR mutagenesis. The sense primer for T138H/A141E and T138H/A141D was 5'-GATC CCGCGAAATTAATACGACTCACTA-3' (28mer, corresponding to pET15b plasmid), whereas the anti-sense primers for T138H/A141E and T138H/A141D were 5'- CTTCCCCTTCTTGGCCTC GAGTTCCTTGTA -3' (30mer, corresponding to Tyr139 - Lys148), and 5'-CTTCCCCTTCTTGGCCT CGAGATCCTTGTA-3' (30mer, corresponding to Tyr139 - Lys148), respectively. The amplified fragment was digested with *Xho* I and *Nco* I, and then inserted into pETTP after digestion with the same endonucleases. The resultant expression vectors for variants were sequenced, and then named as pETTPHE and pETTPHD, respectively.

Preparation of wild type Tth PPase and its Variants.

Purification of wild type *Tth* PPase and its variants was performed according to the previous paper [9]. Briefly, *E. coli* BL21 (DE3) cells transformed with each expression vector for wild type *Tth* PPase and its variants were cultured in 1 liter of LB/Amp medium at 37 ℃ for 20 h. Cells were lysed by sonication, and these soluble fraction was applied on DEAE-cellulose anion-exchange column chromatography followed by Sephacryl S-200HR gel filtration chromatography. Fractions which showed a single band on SDS-polyacrylamide gel electrophoresis were collected, and used as the purified enzyme.

Enzyme Assay

The activity of PPase was assayed at 37°C essentially according to the method described previously [27], the liberation of inorganic phosphate being determined by the method of Peel and Loughman [28]. Protein concentrations were determined by the method of Bradford [29], using bovine serum albumin as the standard.

Thermostability

Enzymes (0.1mg/ml) in 20mM Tris-HCl buffer (pH7.8) were incubated at various temperatures for 1 h, followed by rapid cooling on ice for 10 min. Subsequently, the remaining enzyme activity was measured as described above. The experiment for thermal inactivation was performed by heating each enzyme (0.1mg/ml) at 85℃ for 10, 20, 30, 40, 50, and 60 min. Then, they were rapidly cooled and their enzyme activities were measured as described above.

Circular Dichroism (CD) Spectra Measurements

CD spectra were recorded with a J-720 automatic recording dichrograph (JASCO) at room temperature with protein concentration of 0.1 mg/ml. The far-UV CD spectra were measured between 200 and 250 nm in a 1mm optical path cuvette. CD data are expressed in terms of mean residue ellipticity, [θ], using the mean residue molecular weight from the primary structure.

Fluorescence Spectra Measurements

Fluorescence measurements were made with an F-2500 spectrofluorometer (Hitachi) at room temperature, using a 1 cm path length quartz cuvette. The protein concentration was always adjusted to 0.05 mg/ml in 20mM Tris-HCl buffer (pH 7.8). Tryptophan excitation was performed at 295 nm. The emission spectra were set between 320 and 400 nm.

Samples after heating above 85 ℃ were centrifuged, and each supernatant was measured.

Analysis for Quaternary Structure

Each molecular weight of the wild type *Tth* PPase and its variants was determined by TSKgel G3000SW column (Tosoh) with bed dimensions of 7.5mm I.D. × 30cm. The column was run at room temperature with 50mM Tris-HCl buffer (pH 7.8) as the eluent (flow rate, 0.3 ml/min.). Samples (0.1 mg/ml) were heated at 40, 50, 60, 70, 80, 85 and 90 °C for 1 h, followed by frozen at -20°C, thawed and filtrated with Millex filter (pore size 0.45 mm, Millipore). Then, 5μg of aliquot was injected to the column. The ratio of hexamer/trimer was determined by estimating each peak area of the gel filtration with a Shimadzu Chromatopac C-R6A.

Structure Analysis of Wild type Tth PPase and its Variants.

The hexameric X-ray structural data for *Tth* PPase was obtained from Teplyakov.A. kindly, whereas monomeric data was withdrawn from Protein Data Bank as the PDB code 2PRD. Swiss-PdbViewer ver.4.0 (Swiss Institute of Bioinformatics) was used to analyze structures of wild type *Tth* PPase and intertrimer-converted variants. Then the prime structures of variants were created by "mutate" tool, followed by refined by energy minimization tool with 100 steps of steepst descent to the substituted residues. Gromos96 with 43B1 parameters set was used as force field in Swiss-PdbViewer software [26,30].

RESULTS

As described above, *Tth* PPase, *Sac* PPase, and *E.coli* PPase belong to prokaryotic subfamily in Family I PPase, and resemble in three-dimensional structure and forming a hexamer. However, they differ in thermostability and oligomeric stability. From the comparison of their three-dimensional structures, they might be occurred by the differences in residues and interactions at the intertrimeric interface [21]. Therefore, in order to confirm the relationship between the combination of residues at intertrimeric interface and thermostability, we converted the combination of Thr138 and Ala141 in *Tth* PPase into those of His and Asp/Glu in *E.coli* and *Sac* PPases (namely HD and HE variants, respectively). Additionally, we also prepared T138H and A141E variants of *Tth* PPase, and compared these thermal characteristics in terms of enzyme activity, CD spectra, Trp-excited fluorescence spectra, and quaternary structure.

Initial characterization for intertrimer-converted variants.

In order to estimate the effect of these substitutions on the *Tth* PPase molecule, we analyzed the characteristics of above four variants (HD, HE, T138H, and A141E) in terms of the specific activity, fluorescence spectra and quaternary structure, after the dilution to a concentration of 0.1mg/ml (Table II). The specific activities of HD and HE showed approximately 50% of the activity of the wild type *Tth* PPase, while those of A141E and T138H also decreased to 70 and 58%. Subsequently, to examine whether these decreases in their enzyme activity might be due to structural changes or not, CD and Trp-excited fluorescence spectra was measured (Fig.1 and Table II). In their CD spectra, the drastic perturbation was not observed, and it was deduced that the introduced substitutions might not affect on their secondary structure. Meanwhile, there are two Trp residues (Trp149 and Trp155) in *Tth* PPase, which Trp149 is located in the vicinity of intertrimer interface whereas Trp155 in the internal moiety of molecule. Trp-excited fluorescence spectra may

Table II The characteristics of wild type *Tth* PPase and intertrimer-converted variants.

	Enzymatic activity		Fluorescence spectra		CD spectra		Quaternary structure [e]
	Specific activity (units/mg)	Relative activity [a] (%)	λ_{max} [b] (nm)	F_{max} [c]	$[\theta]_{222nm}$ (deg・cm²/dmol)	α-helix content [d] (%)	
Wild type	890	100	335.0	2770	-8480	20.3	Hexamer
T138H	516	58.0	336.0	3080	-8110	19.0	Hexamer
A141E	622	69.9	335.0	2550	-8520	20.4	Hexamer
T138H/A141E	457	51.3	335.0	3010	-8500	20.3	Hexamer
T138H/A141D	411	46.2	335.0	2070	-8420	20.1	Hexamer

[a] The specific activity of wild type *Tth* PPase was taken as 100%. [b] λmax is the maximum wavelength of tryptophan fluorescence spectra. [c] Fmax is the fluorescence intensity (arbitrary unit) at the emission maximum of tryptophan fluorescence spectra. [d] α - helix content was calculated by the following equation [33]; α - helix content (%) = −([θ]222nm + 2,340) × 100 / 30,300. [e] Quaternary structure was estimated from the elution profile on TSK-gel G3000SW gel filtration chromatography.

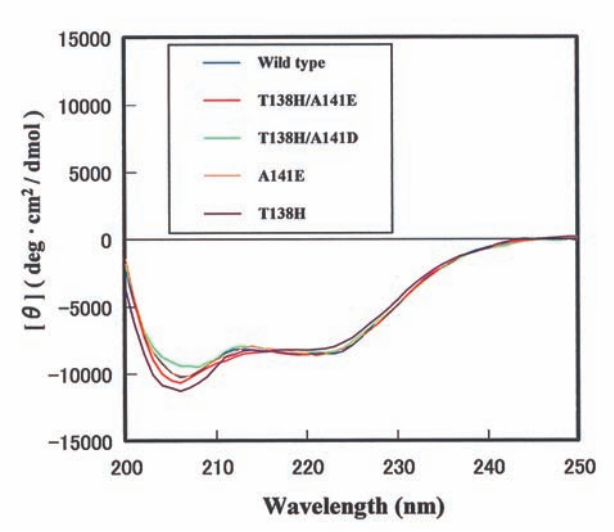

Fig. 1 Far-UV CD spectra of wild type *Tth* PPase and intertrimer-converted variants.

The CD spectra were measured under the conditions described in MATERIALS AND METHODS. The each spectrum for wild type *Tth* PPase and its variants was drawn by the indicated line in Figure.

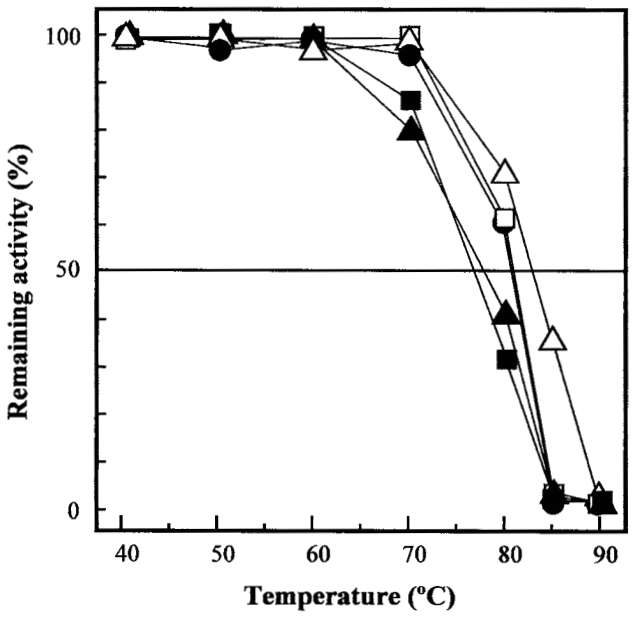

Fig. 2 Thermostability of enzyme activity for wild type *Tth* PPase and intertrimer-converted variants.

The enzyme (0.1mg/ml) was incubated in 20mM Tris-HCl buffer (pH 7.8) at various temperatures for 1 h. Then the enzyme activity was measured at 37℃ after rapid cooling, and the activity at 40℃ was taken as 100%. Symbols: ●, wild type; ▲, T138H; ■, A141E; □, T138H/A141E; Δ, T138H/A141D.

reflect on the environment in the vicinity of two Trp residues. Consequently, we observed a slightly decrease of fluorescence intensity in the HD variant, which Trp residues seemed to be more hydrophilic than that in the wild type. Furthermore, we investigated the quaternary structures of four variants at the concentration of 0.1mg/ml. As results, dissociation of hexamer into trimers was not observed in wild type *Tth* PPase and all variants. Accordingly, it was suggested that the decreases of variants in enzyme activity might not be occurred due to drastic conformational changes or dissociation, but minor changes of active site in the vicinity of these substituted residues.

Thermostabilities of intertrimer-converted variants in enzyme activity.

In native state, though substitutions of residues at intertrimer interface may induce the decrease of enzyme activity, their conformation and quaternary structure seemed not to be perturbed drastically (Fig.1 and Table II). Then, we also examined their thermostabilities in terms of enzyme activity in order to confirm the effect of these substitutions on the active site of enzyme (Fig.2). In contrast to the initial characterization of variants, it was appeared that thermostabilities in the enzyme activity for HD, HE and wild type may be much higher than those of T138H or A141E. In particular, the HD variant is the most thermostable among all enzymes, and remained about 36% (145 units/mg) of the enzyme activity after heating at 85℃ for 1h. On the contrary, either substitution of T138H or A141E might have diminished the thermostability in the enzyme activity, probably by much weaker intertrimeric interactions rather than the wild type *Tth* PPase. Furthermore, we investigated the time course of thermal inactivation for intertrimer-converted variants at 85℃. As shown in Fig.3, the HD variant exhibited about 40% of the native enzyme activity after incubation for 1h, whereas wild type enzyme and other variant almost inactivated rapidly for 10min. Then, it was proved obviously that thermostability of the HD variant has increased in terms of enzyme activity. Therefore, we considered that the introduced His and Asp residues at 138 and 141 positions have increased the thermostability of *Tth* PPase by additive effects of interactions between His138, Asp141 and adjacent residues in the active site of *Tth* PPase.

Conformational thermostabilities of intertrimer-converted variants.

Moreover, we examined conformational thermostability of intertrimer-converted variants by using intrinsic Trp residues (Trp149 and Trp155) as

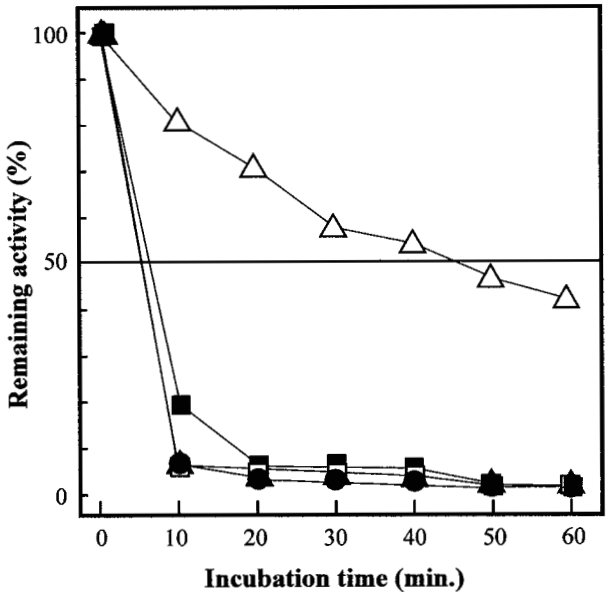

Fig. 3 Thermal inactivation of wild type *Tth* PPase and intertrimer-converted variants on heating at 85°C.

The enzyme (0.1mg/ml) was incubated in 20mM Tris-HCl buffer (pH 7.8) at 85°C for the indicated times. Then the enzyme activity was measured under the same conditions as Figure 1. The enzyme activity without heating was taken as 100%. Symbols: ●, wild type; ▲, T138H; ■, A141E; □, T138H/A141E; Δ, T138H/A141D.

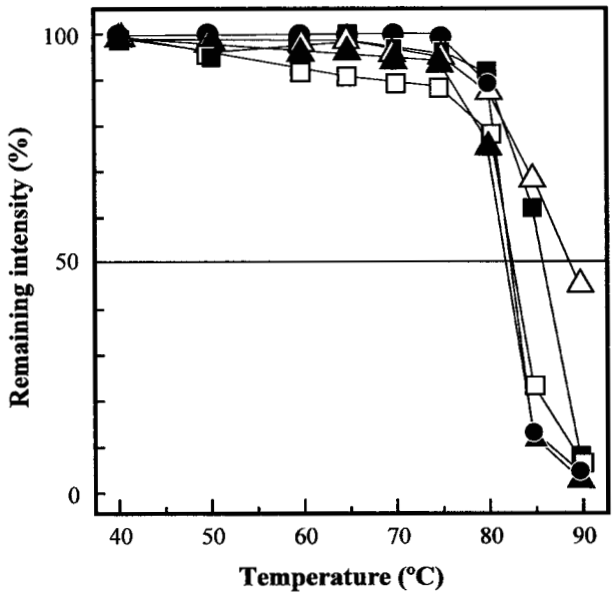

Fig. 4 Thermostability of Tryptophan fluorescence spectra for wild type *Tth* PPase and intertrimer-converted variants.

The enzyme (0.05mg/ml) was incubated in 20mM Tris-HCl buffer (pH 7.8) at the indicated temperatures for 1 h. Then, the emission spectrum of each sample was measured with excitation at 295 nm after rapid cooling. On heating above 85 °C, each sample was centrifuged after heating, and then its supernatant was measured. The fluorescence intensity at maximum wavelength was plotted as relative value to that at 40 °C. Symbols: ●, wild type; ▲, T138H; ■, A141E; □, T138H/A141E; Δ, T138H/A141D.

probes for conformational changes. As shown in Fig.4, HD variant also showed the highest thermostability of all enzymes, especially after heating above 85 °C. Hence, it was proved that conversion to His138 and Asp141 make conformation of *Tth* PPase molecule more thermostable. Meanwhile, HE variant exhibited the slight decrease of thermostability below 80°C relative to wild type enzyme, while slight increase was observed at 85°C. These results seemed to be due to the additive effect of those of T138H and A141E. Interestingly, A141E variant was appeared to be more thermostable than wild type and HE variant at high temperatures. Then, we assumed that the combination of Thr138 and Glu141 might palliate unfavorable steric hindrance at intertrimer interface of *Tth* PPase, as compared with that of His138 and Glu141.

Oligomeric thermostabilities of intertrimer-converted variants.

In previous studies, we reported that hexameric wild type *Tth* PPase is not markedly dissociated but aggregated at high temperatures irreversibly [9,24], whereas *E.coli* and some prokaryotic PPases dissociated into trimers or monomers accompanying with a loss of enzyme activity. Then, we proposed that thermal characteristics of PPases may be defined as non-dissociation and dissociation types. Thus, in order to investigate oligomeric thermostabilities of these intertrimer-converted variants, gel filtration analysis of these PPases was performed after heating at various temperatures. As shown in Figs.5 and 6, HD variant showed the highest thermostability of hexamer of all enzymes, being consistent with above results. On heating even at 85°C, about 38% of hexamer was observed in HD variant (Fig.6). On the contrary, the remarkable changes were observed in oligomeric structure of HE and A141E variants after heating between 40 and 80°C. Obviously, their hexamer would be dissociated into trimers (Fig.5), and almost disappeared above 85°C because of thermal aggregation (Fig.6). In particular, trimers of A141E were more prominent rather than hexamer at 80°C (approximately 60%), whereas ratios of hexamer and trimer of HE variant may be competitive between 60 and 80°C. As compared with Fig.2, it was deduced that some active trimer may exist in HE and A141E variants, since they showed enzyme activities to some extent under the condition occurring the dissociation into trimer. Therefore, it is evident that

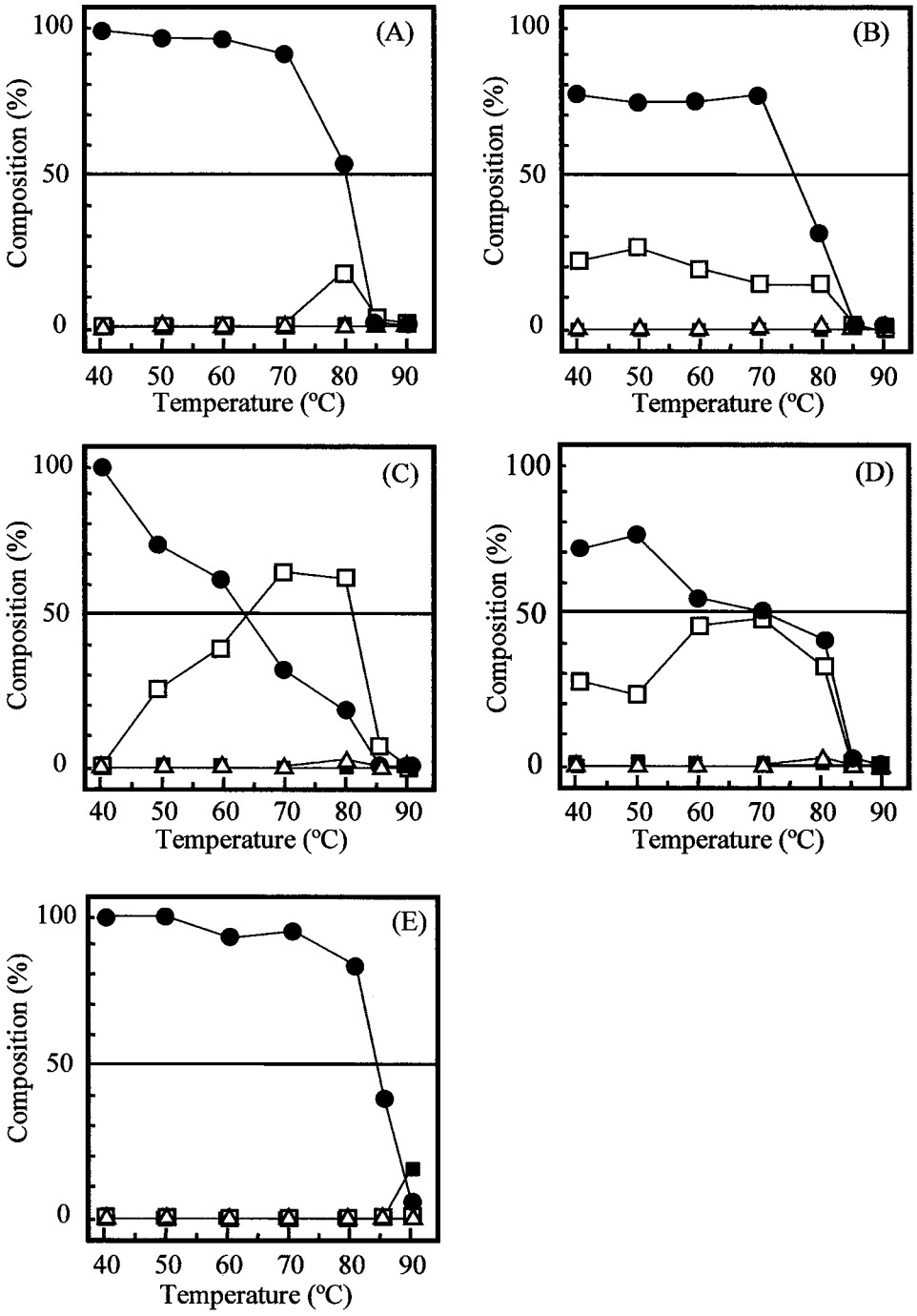

Fig. 5 Oligomeric thermostability for wild type *Tth* PPase and intertrimer-converted variants.

Panels: (A) Wild type, (B) T138H, (C) A141E, (D) T138H/A141E, (E) T138H/A141D. The enzyme (0.1mg/ml) was incubated in 20mM Tris-HCl buffer (pH 7.8) at the indicated temperatures for 1 h. After rapid cooling, the gel filtration chromatography was performed as described in Materials and Methods. Each composition was estimated from each peak area of hexamer, trimer, monomer, and oligomer (component having much higher molecular weight than hexamer) in the HPLC elution profiles. 100% was taken as the sum of all peak area after heating at 40°C. Symbols: ●, hexamer; □, trimer; Δ, monomer; ■, oligomer.

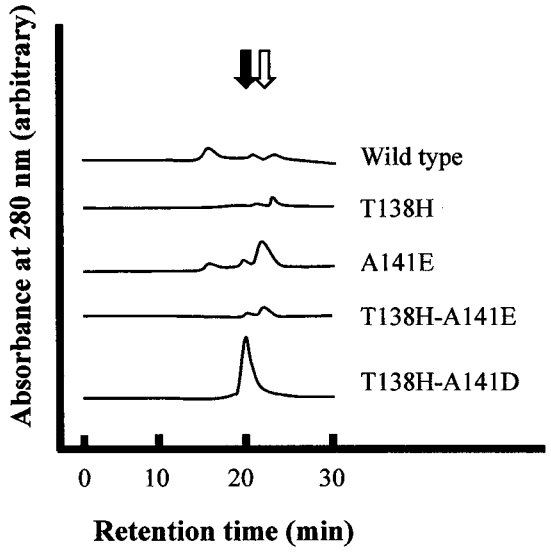

Fig. 6 The elution profiles of TSKgel G3000SW gel filtration chromatography (HPLC) for wild type *Tth* PPase and intertrimer-converted variants after heating at 85°C.

The black and white arrows indicate the retention times for the hexamer and trimers, respectively. Each protein (0.1mg/ml) was heated at 85°C for 1h, and then treated as described in Materials and Methods. 50μl of each sample was injected to column, The flow rate was 0.3 ml/min, and eluted with 50mM Tris-HCl buffer (pH 7.8) at room temperature.

thermal characteristics of quaternary structure should be closely related with features of the side chains at 138 and 141 positions in intertrimer interface of *Tth* PPase. Consequently, the conversion them into the combination of His and Asp is the most suitable for its thermostabilization among various combinations we attempted.

DISCUSSION

As described above, inorganic pyrophosphatases (PPase) in prokaryote subfamily of Family I PPases form hexamer, tetramer, and trimer, and they differ in thermostability and oligomeric interactions [6–8]. To date, three-dimensional structures of hexameric PPases from *E.coli* (*E.coli* PPase), *Thermus thermophilus* (*Tth* PPase), *Sulfolobus acidocardarius* (*Sac* PPase), and *Pyrococcus horikoshii* (*Pho* PPase) have been solved [18, 20-22]. From the comparison between those of *Tth* and *E.coli* PPase, Salminen *et al.* suggested that difference in their thermostability must be caused by some factors like intermonomer interactions, ionic interactions between C-terminus and the rest of molecule, and no Ser and many Arg beyond the end of β7 strand [23]. Furthermore, it was also suggested that intermonomer interaction must be responsible for thermostabilities of *Sac* and *Pho* PPases as same as that of *Tth* PPase [18, 21, 23]. In particular, above four PPases varied in amino acids at intertrimer interface, which is a kind of intermonomer interface. Contacts of this interface are mainly formed by amino acids between α-helix, in which Thr138 and Ala141 in *Tth* PPase are not conserved among prokaryotic PPases (Table I). Additionally, thermal characteristics of *Tth* PPase exhibit a few remarkable features, like non-dissociation and irreversible thermal aggregation. Therefore, we focused on intertrimeric residues, Thr138 and Ala141 in *Tth* PPase, and explored the relationship between these amino acids and thermal characteristics by converting them into the combinations of His and Asp/Glu residues (intertrimer-converted variants; namely HD and HE variants), and substituting Thr138 to His or Ala141 to Glu (T138H and A141E, respectively).

At first, we analyzed some characteristics of intertrimer-converted variants (Table II). The specific activities of four variants were reduced to 69.9% (A141E), 58.0% (T138H), 51.3% (HE) and 46.2% (HD) of wild type enzyme activity, respectively. Meanwhile, from the spectroscopic data and gel filtration (Table II), the drastic conformational change or dissociation of hexamer were not observed in these variants. Additionally, we also measured far-UV CD spectra of these variants, and confirmed no remarkable perturbation on secondary structure (Fig.1). Hence, it was assumed that this reduction of their enzyme activities may not be due to the structural perturbation, but slight conformational changes in the vicinity of active site in *Tth* PPase molecule by these substitutions. Subsequently, we examined thermostabilities of these variants in terms of enzyme activity, in order to confirm effects of substitutions on the active site of enzyme (Figs.2 and 3). As results, HD variants are most thermostable of all enzymes, whereas T138H and A141E decreased their thermostabilities rather than wild type enzyme and HE. Thus, the combination of T138H and A141D substitutions, rather than T138H and A141E, must have stabilized the conformation of active site in *Tth* PPase at high temperature. Likewise, the increased thermostabilities of the HD variant were also observed in the fluorescence spectra and quaternary structure, especially at 85°C (Figs.4, 5, and 6). Thus, it was evident that HD variant would exhibit higher thermostability than wild type *Tth* PPase and other variants, owing to increase of structural integrity and suppression of thermal aggregation (Fig.6).

On the contrary, HE and A141E showed dissociation of hexamer into trimers after heating above 50°C significantly (Fig.5). Interestingly, A141E showed higher conformational thermostability than

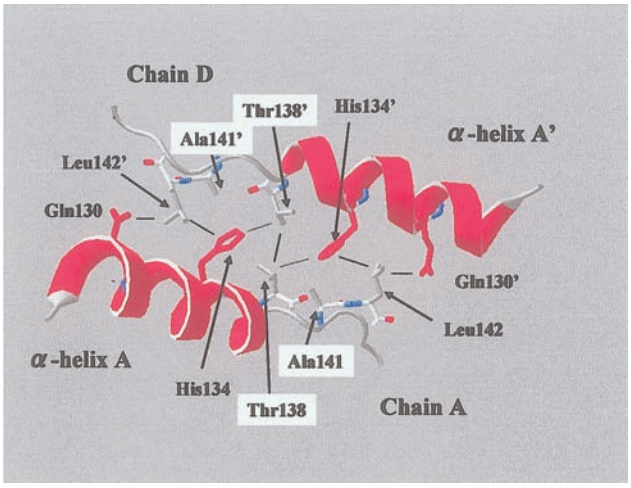

Fig. 7 Intertrimer interface (α-helix A) between monomer A and D in *Tth* PPase.

The figure was drawn by a Molecular Graphics Program, Swiss-PdbViewer Ver.4.0 (Swiss Institute of Bioinformatics). The hexameric X-ray structural data for *Tth* PPase was kindly obtained from Teplyakov.A., whereas monomeric data was withdrawn from Protein Data Bank as the PDB code 2PRD. The stick representations indicate the main and side chains of intertrimer amino acid residues. Hydrophobic contacts are drawn by line, whereas hydrophilic ones by dotted line. The α-helix A contains the residues from Glu126 to Thr138 in *Tth* PPase [20, 23]. The residues without or with prime are located in monomer A and another monomer D, respectively. Thr138 and Ala141 are boxed in this figure.

the HE variant at 80℃, in spite of the dissociation into trimers and the decrease of enzyme activity.

In this disagreement, we deduced that the substitution of Ala141 to Glu might stabilize intratrimeric interactions rather than intertrimeric interactions. Alternatively, such a tendency was also observed in oligomeric thermostability for HE variant. The ratios of hexamer and trimer may be competitive in the HE variant between 60 and 80℃, whereas A141E might tend to dissociate into trimers as temperature increased (Fig.5). As to the reversibility of oligomeric state, we deduced that this reassociation to hexamer by cooling might be at equilibrium rather than reversible completely, because these samples in this experiment were frozen once after heat treatment, followed by thawed and analyzed. If the reassociation to hexamer is reversible, it must be detected by cooling. Therefore, we considered that though the substitution of Ala141 to Glu might induce the dissociation into trimers between 60 and 80℃ by prominent intratrimeric interactions, additional substitution of Thr138 to His could compete alternative intratrimeric or intertrimeric interactions.

On the basis of these investigations, we inferred the results in this study by using computer program Swiss-PdbViewer ver.4.0 [26], and three-dimensional structure withdrawn from Protein data bank (PDB). In wild type *Tth* PPase, it was reported that Nε2 atom of His134 interacts hydrophilically with O atom of Thr138' (in another subunit) at 3.21Å in the intertrimer interface of hexameric *Tth* PPase, while Cγ2 atom of Thr138 interacts hydrophobically with another Cγ2 atom of Thr138' at 3.76 Å. In addition, Cγ atom of His134 interacts hydrophobically with

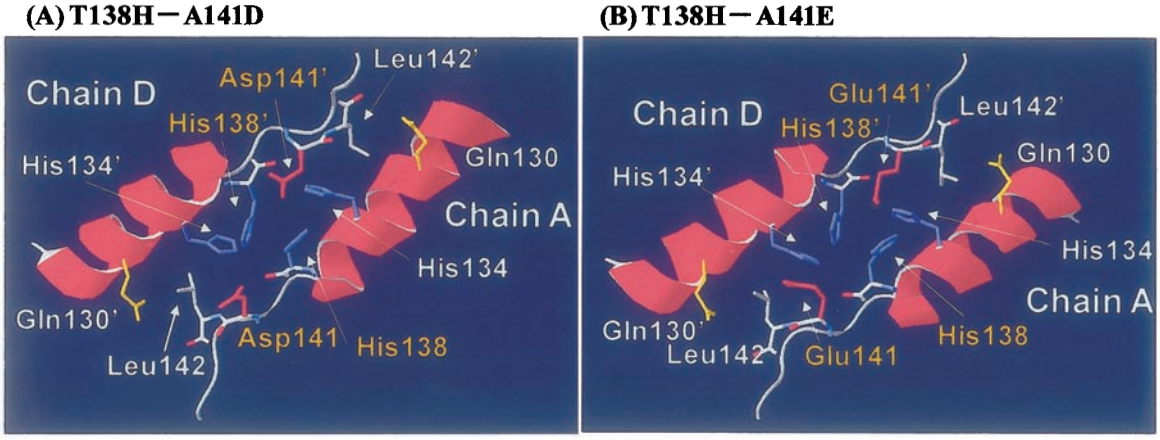

Fig. 8 Molecular modeling of intertrimer interface between monomer A and D in HD and HE variants.

The molecular modeling was performed by Swiss-PdbViewer Ver.4.0 (Swiss Institute of Bioinformatics). Then the prime structures of variants were created by "mutate" tool, followed by refined by energy minimization tool with 100 steps of steepst descent to the substituted residues. The hexameric X-ray structural data for *Tth* PPase was used as template. Gln130 (yellow), His134 (blue), His138 (blue), Asp/Glu141 (red) and Leu142, forming intertrimer interface, are drawn in figure.

Cδ1 atom of Leu142' at 3.83 Å, and Cε atom of His134 with Cδ atom of Gln49' at 3.91 Å in this interface. The trimer-trimer interface in hexameric *Tth* PPase is formed mainly by symmetry-related α-helix A which contains His 134 and Thr 138, while Ala141 may not relate with intertrimeric contacts directly (Fig. 7) [20,23]. Additionally, we estimated the average distance between Ala141 and His134' may be at about 4Å in the intertrimer interface of wild type *Tth* PPase by Swiss-PdbViewer ver.4.0 software. Moreover, we also estimated for HD variant by the molecular modeling, so that the nearest distance between Asp141 and His134' or His138' in intertrimer contacts may be at about 3.6 and 3.3Å respectively, being smaller than the distance between Asp141 and His138 (4.3Å) in intratrimer contacts (Fig. 8 (A)). Conversely, as to HE variant, distance between Glu141 and His134' or His138' in intertrimer had been estimated as about 4.9 and 3.4Å, whereas Glu141 and His138 in intratrimer at 3.8Å (Fig.8 (B)). Then, we considered that wild type *Tth* PPase and HD variant might prefer intertrimeric interactions to intratrimer interaction, while HE variant would prefer intratrimeric interactions by the disruption of intertrimeric interactions conversely. In addition, this intertrimer interface may affect on the thermostability of the enzyme activity at high temperature (Fig.2), because adjacent catalytic amino acids such as Tyr139, Lys140 and Lys148 are located in this intertrimer interface. Therefore, we assumed the possibility that the introduced ionic amino acids and their ionic interactions in the HD variant may increase the solubility and suppress the exposure of internal moiety (including active site) of molecule at high temperature, followed by suppressed the thermal aggregation.

On the other hand, the similar cases of the dissociation of hexamer into trimers by substitution were also reported in H136Q and H140Q variants of *E.coli* PPase, and these substitutions of intertrimeric residues with Gln lead to the disruption of intertrimeric interactions and formation of active trimers [31, 32]. We presumed that these results on *E.coli* PPase might resemble in cases of our HE or A141E variants, so that these substitutions must have occurred the stabilization of intratrimer interactions relative to intertrimer ones because of properties like Gln or Glu residues. Then, we proposed in this study that the HE and A141E variants converted from non-dissociation type to the dissociation type by these effects, though we could not confirm the dissociation of the HD variant.

Consequently, we assumed the possibility in this study that the combination of amino acids at 138 and 141 positions of *Tth* PPase may determine thermal characteristics of intertrimer-converted variants by

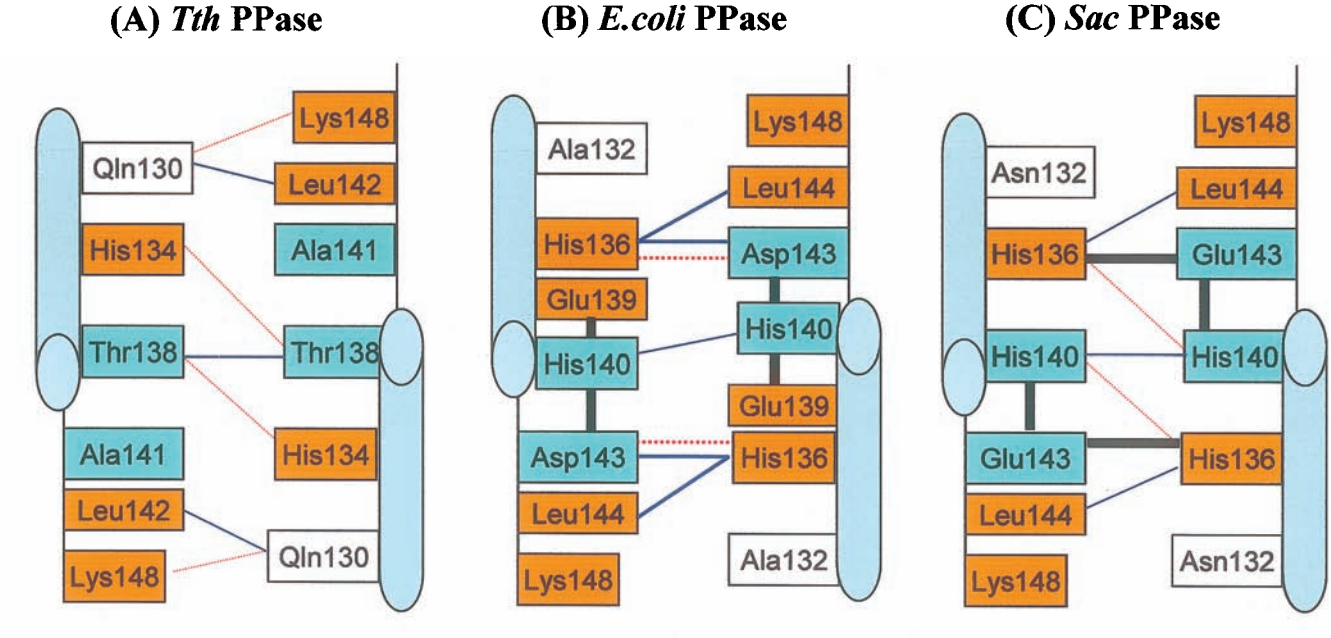

Fig. 9 Schematic representations of intertrimer interface in *Tth*, *E.coli* and *Sac* PPases.

Panels: (A) *Tth* PPase, (B) *E.coli* PPase, (C) *Sac* PPase. Details in these interactions were described in the text and reference 23. Red dotted lines indicate hydrophilic interactions, whereas blue lines show hydrophobic contacts. Black bold lines represent ionic interactions. α–helix A is drawn by cylinder. Amino acids in orange box are conserved among three PPases, while those in this study are highlighted by cyan box. Adjacent Tyr139, Lys140, Lys148 in *Tth* PPase are involved in the active site of the enzyme, which are conserved among three PPases.

either intertrimeric or intratrimeric effects, and the difference in the bulkiness between Glu141 and Asp141 should give rise to the steric hindrance or thermostabilization (Figs.8 and 9). From these points of view, it was deduced to be essential for the oligomeric shift by ionic interactions or the expansion of the intertrimer interface by interactions between the other residues of α-helix A as exemplified in *E.coli* and *Sac* PPase (Fig.9) [23], if we would attempt to thermostabilize the *Tth* PPase molecule by introducing His138 and Glu141.

From these investigations, we conclude that the conversion to a set of hydrophilic His138 and Asp141 had increased the thermostability followed by suppressed its thermal aggregation, because of stabilization of intertrimer interface in *Tth* PPase. Considering the suitable combination of side chains between two α-helices, further investigations to thermostabilize *Tth* PPase are now undertaken by the combination of T138H/A141D in this study and other thermostabilizing factors.

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REFERENCES

[1] Neet, K.E. and Timm, D. E. (1994) Conformational stability of dimeric proteins: Quantitative studies by equilibrium denaturation. *Protein Sci*. **3**, 2167-2174

[2] Buchbinder, J.L., Luong, C.B.H., Browner, M.F., and Fletterick, R.J. (1997) Partial activation of muscle phosphorylase by replacement of serine 14 with acidic residues at the site of regulatory phosphorylation. *Biochemistry* **36**, 8039-8044

[3] Baik, S.H., Michcl, F., Aghajari, N., Haser, R., and Harayama, S. (2005) Cooperative effect of two surface amino acid mutations (Q252L and E170K) in glucose dehydrogenase from *Bacillus megaterium* IWG3 on stabilization of its oligomeric state. *Appl. Environ.Microbiol* **71**, 3285-3293

[4] Ahern, T.J., Casal, J.I., Petsko, G.A., and Klibanov, A.M. (1987) Control of oligomeric enzyme thermostability by protein engineering. *Proc. Natl. Acad. Sci.USA* **84**, 675-679

[5] Lahti, R., Microbial inorganic pyrophosphatases. (1983) *Microbiol. Rev.* **47**, 169-179

[6] Sivula, T., Salminen, A., Parfenyev, A.N., Pohjanjoki, P., Goldman, A., Cooperman, B.S., Baykov, A.A., and Lahti, R. (1999) Evolutionary aspects of inorganic pyrophosphatase. *FEBS letters* **454**, 75-80

[7] Young, T.W., Kuhn, N. J., Wadeson, A., Ward, S., Burges, D., and Cooke, D., *Bacillus subtilis* ORF yybQ encodes a manganese-dependent inorganic pyrophosphatase with distinctive properties: the first of a new class of soluble pyrophosphatase? *Microbiolgy*, **144**, 2563-2571 (1998)

[8] Shintani, T., Uchiumi, T., Yonezawa, T., Salminen, A., Baykov, A.A., Lahti, R, and Hachimori, A. (1998) Cloning and expression of a unique inorganic pyrophosphatase from *Bacillus subtilis*: evidence for a new family of enzymes. *FEBS letters* **439**, 263-266

[9] Satoh, T., Samejima, T., Watanabe, M., Nogi, S., Takahashi, Y., Kaji, H., Teplyakov, A., Obmolova, G., Kuranova, I., and Ishii, K. (1998) Molecular cloning, expression and site-directed mutagenesis of inorganic pyrophosphatase from *Thermus thermophilus* HB8. *J. Biochem.*, **124**, 79-88

[10] Meyer, W., Moll, R., Kath, T., and Schäfer, G. (1995) Purification, cloning, and sequencing of archaebacterial pyrophosphatase from the extreme thermoacidophile *Sulfolobus acidocaldarius*. *Arch.Biochem.Biophys.* **319**,149-156

[11] Wakagi, T., Oshima, T., Imamura, H., and Matsuzawa, H. (1998) Cloning of the gene for inorganic pyrophosphatase from a thermoacidophilic archaeon, *Sulfolobus sp.* strain 7, and overproduction of the enzyme by coexpression of tRNA for arginine rare codon. *Biosci. Biotechnol. Biochem.* **62**, 2408-2414.

[12] Deckert, G, Warren, P.V., Gaasterland, T., Young, W.G., Lenox, A.L., Graham, D.E., Overbeek, R., Snead, M.A., Keller, M., Aujay, M., Huber, R., Feldman, R.A., Short, J.M., Olsen, G.J., and Swanson, R.V. (1998) The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature* **392**, 353-358.

[13] Hoe, H.S., Kim, H.K., and Kwon, S.T. (2002) Cloning, analysis, and expression of the gene for inorganic pyrophosphatase of *Aquifex pyrophilus* and properties of the enzyme. *Mol. Cells*, *13*, 296-301

[14] Lahti, R., Pitkäranta, T., Valve, E., Ilta, I., Kukko-Kalske, E., and Heinonen, J. (1988) Cloning and characterization of the gene encoding inorganic pyrophosphatase of *Escherichia coli* K-12. *J.Bacteriol.* **170**, 5901-5907

[15] Mitchell, S.J. and Minnick, M.F. (1997) Cloning, functional expression, and complementation analysis of an inorganic pyrophosphatase from *Bartonella bacilliformis*.

Can J Microbiol. **43**, 734-743.

[16] Satoh, T., Shinoda, H., Ishii, K., Koyama, M., Sakurai, N., Kaji, H., Hachimori, A., Irie, M., and Samejima, T. (1999) Primary structure, expression, and site-directed mutagenesis of inorganic pyrophosphatase from *Bacillus stearothermophilus*. *J. Biochem,*. **125**, 48-57

[17] Maruyama, S., Maeshima, M., Nishimura, M., Aoki, M., Ichiba, T., Sekiguchi, J., and Hachimori, A. (1996) Cloning and expression of the inorganic pyrophosphatase gene from thermophilic bacterium PS-3. *Biochem. Mol. Biol. Int.* **40**, 679-688

[18] Liu, B., Bartlam, M., Gao, R., Zhou, W., Pang, H., Liu, Y., Feng, Y., and Rao, Z. (2004) Crystal structure of the hyperthermophilic inorganic pyrophosphatase from the archaeon *Pyrococcus horikoshii*. *Biophys. J.* **86**, 420-427,

[19] Richter, O.M.H. and Schäfer, G. (1992) Cloning and sequencing of the gene for the cytoplasmic inorganic pyrophosphatase from the thermoacidophilic archaebacterium *Thermoplasma acidophilum*. *Eur. J. Biochem.* **209**, 351-355

[20] Teplyakov, A., Obmolova, G., Wilson, K.S., Ishii, K., Kaji, H., Samejima, T., and Kuranova, I. (1994) Crystal structure of inorganic pyrophosphatase from *Thermus thermophilus*. *Protein Sci.* **3**, 1098-1107

[21] Leppänen, V. M, Nummelin, H., Hansen, T., Lahti, R., Schäfer, G., Goldman, A. (1999) *Sulfolobus acidocaldarius* inorganic pyrophosphatase: structure, thermostability, and effect of metal ion in an archael pyrophosphatase. *Protein Sci.* **8**, 1218-1231

[22] Harutyunyan, E. H., Oganessyan, V. Y., Oganessyan, N. N., Avaeva, S. M., Nazarova, T. I., Vorobyeva, N. N., Kurilova, S. A., Huber, R., and Mather, T. (1997) Crystal structure of holo inorganic pyrophosphatase from *Escherichia coli* at 1.9Å resolution. Mechanism of hydrolysis. *Biochemistry*, **36**, 7754-7760

[23] Salminen, T., Teplyakov, A., Kankare, J., Cooperman, B.S., Lahti, R., and Goldman, A. (1996) An unusual route to thermostability disclosed by the comparison of *Thermus thermophilus* and *Escherichia coli* inorganic pyrophosphatases. *Protein Sci*, **5**, 1014-1025

[24] Satoh, T., Takahashi, Y., Oshida, N., Shimizu, A., Shinoda, H., Watanabe, M., and Samejima, T. (1999) A chimeric inorganic pyrophosphatase derived from *Escherichia coli* and *Thermus thermophilus* has an increased thermostability. *Biochemistry*, **38**, 1531-1536

[25] Satoh, T., Oshida, N., Ono, M., Hattori, M., Ohta, T., Watanabe, M., Shinoda, H., Takahashi, Y., Lee, J.S., and Samejima, T. (1999) Deletion of Ala144-Lys145 in *Thermus thermophilus* inorganic pyrophosphatase suppresses thermal aggregation. *J.Biochem.* **125**, 858-863

[26] Guex, N. and Peitsch, M.C. (1997) SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophoresis* **18**, 2714-2723.

[27] Hachimori, A., Takeda, A., Kaibuchi, M., Ohkawara, N., and Samejima, T. (1975) Purification and characterization of inorganic pyrophosphatase from *Bacillus stearothermophilus*. *J.Biochem.*, **77**, 1177-1183

[28] Peel, J.L. and Loughman, B.C. (1957) Some observations on the role of copper ions in the reduction of phosphomolybdate by ascorbic acid and their application in the determination of inorganic orthophosphate. *Biochem.J.*, **65**, 709-716

[29] Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-254

[30] W.F. van Gunsteren, S.R. Billeter, A.A. Eising, P.H. Hünenberger, P. Krüger, A.E. Mark, W.R.P. Scott, I.G. (1996) Tironi Biomolecular Simulation: The GROMOS96 Manual and User Guide, Vdf Hochschulverlag AG an der ETH Zürich, Zürich, Switzerland, pp. 1-1042

[31] Baykov, A.A., Dudarenkov, V.Y., Käpylä, J., Salminen, T., Hyytiä, T., Kasho, V.N., Husgafvel, S., Cooperman, B.S., Goldman, A., and Lahti, R. (1995) Dissociation of hexameric *Escherichia coli* inorganic pyrophosphatase into trimers on His-136→Gln or His-140→Gln substitution and its effect on enzyme catalytic properties. *J. Biol. Chem.*, **270**, 30804-30812

[32] Velichko, I. S., Mikalahti, K., Kasho, V. N., Dudarenkov, V. Y., Hyytiä, Goldman, A., Cooperman, B. S., Lahti, R., and Baykov, A. A. (1998)Trimeric inorganic pyrophosphatase of *Escherichia coli* obtained by directed mutagenesis. *Biochemistry*, **37**, 734-740

[33] Chen, Y. H., Yang, J. T., and Matinez, H. M. Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry*, **11**, 4120-4131 (1972)